Research article

The flow of incoming nectar through a honey bee (*Apis mellifera* L.) colony as revealed by a protein marker

G. DeGrandi-Hoffman[1] and J. Hagler[2]

[1] Carl Hayden Bee Research Center, USDA-ARS, 2000 East Allen Road, Tucson, AZ 85719, USA, e-mail: GDHOFF@AOL.COM
[2] Western Cotton Research Laboratory, USDA-ARS, 4135 E. Broadway Road, Phoenix, AZ 85040, USA, e-mail: HAGLERJ@PRIMENET.COM

Summary. The flow of incoming nectar in honeybee (*Apis mellifera* L.) colonies was simulated by feeding a sucrose solution labeled with a novel protein (rabbit IgG) marker and then analyzing bee and colony samples using an enzyme-linked immunosorbant assay (ELISA). The labeled sucrose solution was quickly transported to food storage and brood combs. Within 2h, equal percentages of worker bees from food storage combs, nurse bees and nectar samples tested positive for the marker. Percentages of nurse bees and larvae testing positive also were equal within the first 2 h of feeding it to a colony and these percentages increased over time. Our results suggest that workers with nectar loads deposit them into cells on either food storage or brood comb with equal frequency. The labeled sucrose solution transported to the brood comb is subsequently used by nurse bees to feed larvae. How the deposition of incoming nectar in brood comb might possibly integrate the activities of foragers and nurse bees is discussed.

Key words: Honey bees, trophallaxis, nectar storage, communication, marking techniques, ELISA.

Introduction

Honey bee (*Apis mellifera* L.) colonies are comprised of thousands of individual bees performing different tasks that must somehow be coordinated for the colony to survive. At any given time, different groups of worker bees are rearing brood, building comb, defending the hive, maintaining brood nest temperatures, and collecting and processing nectar and pollen. While it is important for workers performing the same task to work in a coordinated manner, it is also important that those doing complementary tasks coordinate their efforts. A case in point is workers that collect nectar, those that store it in the combs, and those that rear brood.

Nectar is collected from flowering plants by adult worker bees. When nectar foragers return to their colonies from the field, they give their loads to nestmates at the colony entrance (i.e., receiver bees (Seeley, 1992)). Receiver bees transfer the nectar to other nestmates who continue to pass it on until ultimately the nectar is placed in a cell on a comb somewhere in the hive (Seeley, 1992). During the spring and summer, large quantities of nectar are collected and eventually converted into honey that is stored for later use when plants are not blooming or weather is not suitable for foraging. Combs with just honey or nectar often are found above or adjacent to the brood nest. Nectar also is placed in cells on combs where brood is reared. Placing nectar in cells of comb containing brood makes it readily available to nurse bees who feed these resources to the larvae.

Nixon and Ribbands (1952) first described the transfer of nectar from foragers to nestmates in a colony. Within 3.5 h after releasing just six foragers that were fed ^{32}P labeled sugar water into a colony, most of the other foragers (62%) and about a fifth of the worker population in the brood area received some of the labeled food. The rapid transfer of nectar from foragers to other nestmates indicates the numerous trophallactic contacts made among workers in a hive. The exchange of nectar among workers in some cases, is for food processing and storage. However, the radioactive marker was also detected in nurse bees within 4 h of releasing the labeled foragers. This suggests that some incoming nectar is disseminated to combs with brood. The transfer of incoming nectar to cells around the brood might help to coordinate the behaviors of nestmates involved in nectar collection, storage, and rearing brood.

Since nectar is necessary to rear larvae (Ribbands, 1953), it seems likely that some type of information flow might exist between nectar foragers and nurse bees. Eggs and young larvae are eaten rather than reared if colony resources are limited (Weiss, 1984). A communication link has been described between pollen collectors and nurse bees (Camazine, 1993;

Camazine et al., 1998). Unlike pollen foragers though, nectar foragers and nurse bees do not contact each other directly to exchange information about the status of nectar stores in the colony. Consequently, the flow of information from nectar foragers to nurse bees might be through trophallactic contacts between receiver and nurse bees. Alternatively, incoming nectar might be placed into cells of brood combs. How quickly cells with nectar on brood comb are replenished could communicate its availability to nurse bees. If nectar is used at a rapid rate due to vigorous brood rearing, receiver bees would consistently find an area in the hive to unload incoming nectar. Thus, a communication link could be established between workers collecting and storing nectar and those rearing brood that is a by-product of receiver bees finding space for incoming nectar in the cells around the brood area.

The purpose of this study was to describe the flow of incoming nectar through a colony. To accomplish this, we used a novel protein marking technique and enzyme linked immunosorbant assay (ELISA) (Hagler, 1997a, b). We fed sucrose solution labeled with the protein (rabbit IgG) to colonies, and then sampled worker bees on food storage frames, those feeding brood (i.e., nurse bees), larvae, and nectar over time. We then assayed for the presence of the protein using an anti-rabbit IgG. Our findings are interpreted within the context of how the nurse bees and foragers might integrate their activities by the placement and use of the incoming nectar.

Materials and methods

Testing for the presence of rabbit protein fed to honey bees

Preliminary tests were conducted to determine whether rabbit IgG protein could be detected through trophallactic interactions. In these tests, caged workers fed on a 40% sucrose solution containing 1.0 ml of rabbit protein (5.0 mg) (Sigma Chemical Co., St. Louis, MO, No. I8140). As a control, an identical cage was set up and worker bees fed on a 40% sucrose solution without the rabbit protein. The worker bees fed on the sucrose solutions for 24 h *ad libitum*. Then, the feeding jar was removed and 30 worker bees that had not fed on the labeled solution were marked with a white dot on their thorax and were released into each cage. Twenty-four hours later, marked and unmarked bees from cages where the protein was or was not fed to the workers were sampled and placed in separate vials and frozen (–70 °C) until analyzed for the protein marker by ELISA (see below). In these preliminary tests, 100% of the bees exposed to the protein labeled sucrose solution either through feeding or via trophallaxis tested positive for the presence of the protein.

Experiments with observation colonies

Two experiments with different sampling schemes were used in the study. Twelve different European honeybee colonies (4 colonies per trial; 3 trials per sampling scheme) were used as source material for the observation hives in each sampling scheme. The source colonies had surplus food and space available for colony expansion and storage of incoming resources. Bees foraged freely prior to removing frames for the observation colonies. Two frames from each colony were used to make the observation colonies used in the study. Observation colonies were composed of an egg laying queen and two standard depth frames

with drawn comb (43.2 cm × 20.3 cm × 3.8 cm) covered with adult worker bees (approximately 2200 adult honey bees per colony). The lower frame was a typical brood frame containing nectar and pollen along with honey bee eggs, larvae and capped brood in all stages of development. The frame had about 25% of the cells open and available at the start of the study for additional brood or food stores. The upper frame had nectar, capped honey, and open cells (about 50% of the area on the comb) where more nectar could be stored. Frames were selected so that each colony would contain nearly equivalent amounts of larvae, nectar and pollen stores, and open comb. After the observation colonies were established, a clear plastic feeding jar containing a 40% sucrose solution was placed on top over a screen mesh so that the bees could feed from the jar. Three – four days later, the plain sucrose solution was replaced with a protein "spiked" 40% sucrose solution. Care was taken that none of the solution was spilled on to the bees below. Thus, we could be certain that all the samples that tested positive were because of trophallactic contact or nectar storage. The sucrose solution was fed to the colonies *ad libitum*. The amount of solution entering each hive over time was estimated by the amount of sucrose solution remaining in the jar. The observation hives were located in a greenhouse and the sucrose solution we provided was the only available food source.

In the first experiment, the labeled sucrose solution was made available to the colonies at 1700 hrs for 16 h before sampling began. Colonies were sampled at 4 h intervals for 8 h. This experiment determined if the labeled nectar could be detected in the colony if ample time was provided for its translocation to the various areas we targeted for sampling. Based upon the results, a second set of experiments was conducted where the spiked sucrose solution was placed on each colony at 900 hrs and sampled every 2 h afterwards for 8 h. In all instances, colonies were sampled simultaneously by 4 observers (1 observer per colony). During every sampling interval, we collected four samples per colony of each of the following: (1) larvae of all ages, (2) nectar from cells on the brood comb, (3) nurse bees, (4) uncapped cells containing nectar from the food storage comb, and (5) worker bees from the food storage comb. Nurse bees were identified as those workers that were seen consistently inserting their heads into cells containing larvae. Worker bees from the food storage comb were not performing any specific task at the time they were sampled. The top and bottom frames in each observation hive were divided into four equal sections and samples were taken from each section during each sampling interval. The glass panels on the observation hives could be removed before sampling with minimal disturbance to the bees on the frames. This enabled us to reach different areas of the combs throughout the sampling period. To avoid cross-contamination by the marking protein, separate pipettes were used for each nectar sample. The forceps used to remove bees or larvae were dipped in soapy water and rinsed between each sample to destroy any protein that might be on them. Each sample was placed in an individual vial and frozen (–70 °C) until analyzed.

The ELISA procedure

Frozen samples were individually homogenized in 1.0-ml of Tris buffered saline (TBS) (pH = 7.2), and assayed for the presence of the rabbit protein. A sandwich ELISA was performed on the individual samples from the preliminary tests and observation colonies using previously described techniques for marking minute parasitoids (Hagler and Jackson, 1998). Each well of a 96-well ELISA microplate was coated with 100-μl of anti-rabbit IgG (developed in goat) (Sigma Chemical Co., St. Louis, MO, No. R2004) diluted 1 : 500 in ddH$_2$O and incubated overnight at 4 °C. The IgG antibodies were discarded and 360-μl of 1% nonfat dry milk in ddH$_2$O was added to each well for 30 min at 27 °C to block any remaining non-specific binding sites on the plates. After the nonfat milk was removed, a 150-μl aliquot of the homogenized sample was placed in each well of the pretreated assay plate and incubated for 2 h at 27 °C. The samples were then discarded and each well was briefly rinsed three times with TBS Tween 20 (0.05%) and twice with TBS. Aliquots (50-μl) of anti-rabbit IgG conju-

gated to horseradish peroxidase (Sigma, No. A-6154) diluted to 1:1,000 in 1.0% nonfat milk, were added to each well for 1 h at 27°C. Plates were again washed as described above and 50-μl of substrate was added using the reagents supplied in a horseradish peroxidase substrate kit (Bio-Rad, Richmond, CA, No. 172-1064). Following substrate incubation (1 h), the quantitative outcome (optical density) of each sample was measured with a microplate reader set at 405 nm. The mean (± s.d.) ELISA optical density value (quantitative outcome) and the percentage of samples (qualitative outcome) scoring positive for protein were tallied. Samples known not to contain any rabbit protein were assayed by the sandwich ELISA described above. Eight negative controls and 4 TBS blanks accompanied each ELISA plate. Each ELISA plate was "zeroed" using the TBS blanks.

Statistical analysis

The samples from the preliminary tests and observation colonies were scored positive for the presence of rabbit protein if the ELISA optical density value exceeded the mean negative control reading by three standard deviations (Hagler, 1997a, b). The percentage of samples testing positive for the protein was pooled for all colonies and replications in each sampling scheme. The data were arcsin transformed before analysis to stabilize the variance. An arc sin transformation was used because the data were expressed as percentages (Sokal and Rohlf, 1995). Comparisons were made among the percentage of positive samples obtained among the different types of samples over the four sample intervals. If means differed significantly, a Fishers Least Significance Difference test was conducted (Sokal and Rohlf, 1995).

Results

All observation hives in both sampling schemes collected at least 100 ml of the labeled sucrose solution during the course of the sampling period. In the first experiment where colonies were exposed to the solution for 16 h before sampling, there were no significant differences in the percentage of samples testing positive within or among any set of samples (Table 1). About 42–70% of the nurse bees, larvae and nectar from cells around the brood tested positive for the marker as did about 70–75% of the worker bees from the food storage combs. Nectar from the top comb was not sampled in the first sampling scheme.

In the second experiment, the labeled sucrose solution was detected in some samples from all groups of workers and regions of the hive within 2 h after feeding it to a colony (Table 2). Equal percentages of nurse bees and worker bees from the food storage comb tested positive for the protein marker during each sampling interval, as did nectar samples from food storage and brood combs. Percentages of nurse bees, larvae, and nectar from brood comb testing positive also were equal for all sampling intervals. Larvae had the lowest percentage of positive samples during the first and last sampling intervals. The percentages of worker bees and nectar testing positive increased over time, and by 8 h of exposure to the sucrose solution, nearly all samples with the exception of larvae tested positive.

Table 1. Mean percentage of samples testing positive by ELISA for rabbit IgG protein in sucrose solution in Experiment-1 as a function of hours of exposure to honey bee colonies

Sample type	Mean % of positive samples			F	p
	16 h	20 h	24 h		
Nectar from brood comb	41.7 ± 9.9	64.6 ± 11.3	72.7 ± 11.9	2.10	0.13
Worker bees from food storage combs	75.0 ± 8.7	75.0 ± 10.2	72.7 ± 9.8	0.02	0.98
Nurse bees	66.7 ± 10.8	68.7 ± 12.7	59.1 ± 12.7	0.17	0.84
Larvae	62.5 ± 10.4	66.7 ± 10.8	65.9 ± 12.7	0.04	0.96

Means among sample types did not differ significantly as determined by analysis of variance; F = 0.65; p = 0.89. Means are based upon a total of 48 samples (12 samples from 4 colonies) of either bees, larvae, or nectar.

Table 2. Mean percentage of samples testing positive by ELISA for rabbit IgG protein in sucrose solution in Experiment-2 as a function of hours of exposure to honey bee colonies

Sample type	Mean % positive samples ± S.E.			
	2 h	4 h	6 h	8 h
Nectar – top frame	58.3 ± 12.4 ab	66.7 ± 8.9 ab	83.3 ± 7.7 ace	85.4 ± 5.7 ace
Nectar – brood frame	43.7 ± 12.7 bd	72.9 ± 9.9 abc	81.2 ± 9.8 ace	87.5 ± 7.2 ace
Worker bees from food storage combs	62.5 ± 9.5 ab	83.3 ± 7.1 ace	91.7 ± 4.7 ce	97.9 ± 2.1 e
Nurse bees	41.7 ± 6.4 bd	58.3 ± 9.9 abd	81.2 ± 9.3 ace	89.6 ± 4.8 ce
Larvae	30.5 ± 9.0 d	56.2 ± 10.3 ab	70.8 ± 8.0 abc	70.8 ± 10.1 abc

Means followed by the same letter are not significantly different at the 0.05 level by analysis of variance (F = 4.33, df = 19, 220; p < 0.0001) followed by a Fishers least significant difference test. Means are based upon a total of 48 samples (12 samples from 4 colonies) of either bees, larvae, or nectar.

Discussion

In this study, incoming sugar solution was translocated to brood and food storage combs with equal frequency. At least some of the sucrose solution stored in the cells around the brood was used to feed larvae soon after it was collected. Equal percentages of nurse bees, larvae, and nectar from brood comb tested positive for the marker within 2 h after feeding it to the colony. Nurse bees and workers collected from food storage frame also tested positive with equal frequencies suggesting that nurse bee might also have obtained some incoming nectar directly from nestmates.

This study represents the first time a protein has been used to mark the flow of resources in a social insect colony. Others have used protein markers and the ELISA technique to monitor the dispersal of insect herbivores, predators, and minute parasites (Hagler et al., 1992; Hagler and Durand, 1994; Hagler, 1997 a, b; Hagler and Jackson, 1998). Protein labeling of insects and the subsequent analysis for the protein marker by ELISA (or any immunoassay) represents a significant paradigm shift in the way marking studies are conducted (Hagler, 1997). This technique can be used to examine various components of honey bee and other social insect colonies and offers many practical advantages over previously used marking techniques such as radioactive labels (e.g., Nixon and Ribbands, 1952; Crailsheim, 1992; Camazine et al., 1998). The protein marker and the immunoreagents needed for the ELISA are relatively inexpensive, easily obtainable, and no hazardous waste is accumulated. The sandwich ELISA is very sensitive and specific, and the marking ELISA is simple, rapid and safe. Finally, the equipment needed to conduct the ELISA is relatively inexpensive and common in most laboratories. In this study, we used an ELISA microplate reader to quantify the ELISA responses; however, the qualitative nature of this assay combined with the clarity of the negative controls (i.e., no background coloration) may not warrant the purchase of one. A limitation of the ELISA technique is that it provides only qualitative (i.e., yes-no) data while radioactive tracing provides both qualitative and quantitative information.

We fed the labeled sucrose solution to the colonies using feeding jars placed on top of the colonies rather than letting the bees collect it from feeders. We chose to use feeding jars so that we could determine the amount of sucrose solution entering a colony in case few or none of the samples tested positive for the marker. Even though we used a different method to get the labeled sucrose solution into the observation hives, our results were similar to those previously reported by Nixon and Ribbands (1952), who fed radioactive sugar solution to foragers and then released them in full-sized colonies.

The results of our study indicate that incoming nectar might be translocated to cells around the brood nest. However, the results do not connote that brood levels directly regulate nectar foraging or vice versa. Indeed studies have shown that foraging for nectar is not affected by the amount of brood in the colony (Eckert et al., 1994; Fewell and Winston, 1992). Our results do suggest however, that a link between foragers and nurse bees could exist as a by-product of where incoming nectar is placed. The link would be established by receiver bees finding space to deposit incoming nectar in the cells of brood comb. Space would be available there because the nectar is being fed to the larvae. Finding space to deposit incoming nectar causes receiver bees to continue accepting nectar loads which stimulates continued foraging (Seeley, 1992). When space is no longer available to store nectar, receiver bees stop accepting nectar loads. If foragers cannot find receiver bees to accept the nectar they collected, foraging stops (Seeley et al., 1996). Steady replenishment of cells with nectar could enable nurse bees to assess nectar availability in the colony. Thus, by emptying and filling cells on brood comb, foragers and nurse bees might coordinate their behaviors via the placement of nectar loads by receiver bees.

The protein marking and ELISA technique used in this study show that incoming nectar is distributed on both food storage and brood combs and throughout members of a honey bee colony including the larvae in as little as 2 h after it is collected. However, definitive evidence of the direct transport of incoming nectar to brood comb and its influence on the integration of nectar foraging and brood rearing requires additional studies. Two factors make it possible that our findings might represent what happens in larger colonies at least during the early stages of their development. First, colonies are established beginning with the construction of brood comb and any incoming surplus nectar is stored there. Separate food storage combs are added later as the colony expands. Placing some incoming nectar in brood comb as the colony grows might simply be a continuation of a process that was started while the colony was being established. Secondly, when a colony is re-establishing the division of labor after swarming or absconding, it is critical that foragers and nurse bees coordinate their activities. At this time, there are little or no surplus resources and space is limited because comb needs to be built. Thus, some communication link must exist between nurse bees and foragers to avoid starvation. The link might be the receiver bees who either pass nectar loads directly to nurse bees or store them in cells around brood. Future studies will be directed at determining the flow of incoming nectar through full sized colonies during and after their establishment. This might confirm whether strategies to coordinate the behaviors of nectar foragers and nurse bees revolve around the placement of incoming nectar, and if those strategies change or are even necessary after a colony is established and surplus nectar is available.

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